Volume 251, number 1,2, 137–142 FEB 07385 July 1989

The cytosolic G-protein α-subunit in human neutrophils responds to treatment with guanine nucleotides and magnesium

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Received 23 May 1989

The hydrodynamic properties of the G_{i2} α-subunit in human neutrophil cytosol were analyzed. The $s_{20,w}$ value was 3.3 S at 30°C and 4.1 S at 4°C. Reconstitution of the cytosolic α-subunit with βγ-complex purified from porcine brain resulted in an $s_{20,w}$ value of 4.0 S at 30°C and 4.2 S at 4°C. Treatment of cytosol with G-protein-activating agents, GTPγS and $MgCl_2$, decreased the $s_{20,w}$ value to 2.4 S at 30°C and 2.9 S at 4°C. Our results indicate that the cytosolic α-subunit of neutrophils is an inactive monomeric species at 30°C capable of interacting with G-protein βγ-complex and responsive to treatment with activating agents leading to the two activated forms, α* at 4°C and α** at 30°C.

Signal transduction; Guanine nucleotide binding regulatory protein; Guanine nucleotide; Neutrophil; Cytosol

1. INTRODUCTION

Guanine nucleotide-binding proteins (G-proteins) are involved in transmembranous signalling, mediating between activated transmembranous receptors and effectors which generate intracellular signals [2]. In detergent-containing solutions, G-proteins dissociate into α-subunits and βγ-complexes following activation with poorly hydrolyzable GTP analogues in the presence of magnesium [3,4]. Recent evidence suggests that hormonal activation of G-proteins may also lead to the dissociation into α-subunits and βγ-complexes within native membranes [5].

Analyzing heterotrimeric G-proteins in detergent-containing solutions, Codina et al. [4] showed that activation and dissociation of G-proteins can be individually identified and are not necessarily linked. Though others [6–8] have described hydrodynamic properties of isolated G-protein α-subunits, effects of guanine nucleotides and magnesium on hydrodynamic parameters of isolated α-subunits have not been reported.

In cytosol of human neutrophils, a pertussis toxin-sensitive 40 kDa G-protein α-subunit was found [9–12], which has been identified as the G_{i2} α-subunit [13]. It has been shown that neutrophil plasma membranes contain an excess of α- over β-subunits, which may be responsible for the presence of α-subunits in the cytosol [11]. To our knowledge, no successful purification of the cytosolic G_{i2} α-subunit has been reported so far.

In order to obtain information on the structural and functional state of the cytosolic G-protein α-subunit in human neutrophils, we determined its hydrodynamic properties using a crude cytosolic preparation which was devoid of G-protein β-

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Abbreviations: GTPγS, guanosine 5′-*O*-(3-thiotriphosphate); GMP-P(NH)P, guanyl-5′-yl imidodiphosphate; SDS-PAGE, SDS-polyacrylamide gel electrophoresis; pertussis toxin (islet-activating protein), main exotoxin of *Bordetella pertussis*; $s_{20,w}$, standard sedimentation coefficient in water at 20°C

Results have been presented in part at the Spring Meeting of the Deutsche Gesellschaft für Pharmakologie und Toxikologie from 14–17 March 1989 in Mainz, FRG [1], and are part of the thesis of U.R. submitted to the Medical Faculty of the Freie Universität Berlin in partial fulfillment of the requirements for a Doctor of Medical Science degree

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00145793/89/$3.50

subunits as judged by the immunoblotting technique [13].

2. MATERIALS AND METHODS

2.1. *Sucrose density gradients*

Sucrose density gradient centrifugations were performed as described [13] with the following modifications. At 4°C, centrifugation was performed in a Kontron TST 55.5 rotor (40000 rpm, 200000 × g, 15.5 h). When H_2O was replaced by 94% (v/v) D_2O, the centrifugation time was 55–70 min in a Sorvall TV 865 rotor (65000 rpm, 400000 × g, 30°C).

If not indicated otherwise, fractionated samples containing α-subunits only were incubated with purified $\beta\gamma$-complex overnight as the $\alpha\beta\gamma$-heterotrimer is supposed to be the preferential substrate for pertussis toxin [14]. If appropriate, samples were incubated overnight with EDTA to complex free Mg^{2+} which greatly inhibits pertussis toxin-catalyzed ADP-ribosylation [15] and with GDP to displace bound GTPγS. Following the pretreatment, pertussis toxin-catalyzed [^{32}P]ADP-ribosylation of samples was performed as described [15] with modifications. The toxin was preactivated in the presence of 1 mM ATP and 20 mM dithiothreitol. The assay was performed in the presence of Lubrol PX (0.3%, w/v) with the preactivated toxin being employed at a concentration of 2.2 μg/ml. The NAD concentration was 0.3–1 μM with 30–50 kBq [^{32}P]NAD/assay tube. Prior to gel electrophoresis, proteins were precipitated by acetone; the precipitate was washed with trichloroacetic acid and subsequently with methanol or a chloroform/methanol (1:1) mixture.

2.2. *Gel filtration analysis*

Gel filtration was performed using a 7.5 mm × 600 mm (26.5 ml) TSK G3000SW column (LKB, Gräfelfing, FRG); the flow rate was 0.5 ml/min. The column was equilibrated with a buffer consisting of 1 mM Na-EDTA, 1 mM dithiothreitol, 100 mM NaCl and 20 mM Na-Hepes (pH 7.4). Cytosol (130 μl, corresponding to 120 μg protein) was loaded on the column after mixing with 20 μl marker proteins. Fractions (200 μl) were collected and analyzed in the same manner as were the gradient fractions. The Stokes' radii of the employed markers are: catalase, 5.21 nm; fumarase, 5.27 nm; malic dehydrogenase, 3.69 nm and cytochrome *c*, 1.87 nm.

2.3. *Miscellaneous*

Neutrophil cytosol was prepared as described [13]. G-protein $\beta\gamma$-complex was purified from porcine brain as described [16]. The preparation used was devoid of α-subunits as judged by Coomassie Blue-stained SDS gels. Appropriate controls were performed to ensure that the $\beta\gamma$-complex added to the samples did not contain detectable amounts of [^{32}P]ADP-ribosylatable pertussis toxin substrates. Pertussis toxin was a kind gift of Dr Motoyuki Yajima (Shiga, Japan). Lubrol PX was deionized with a mixed-bed ion exchange resin (AG 501-X8, 100–200 mesh, Bio-Rad, München, FRG). [α-^{32}P]ATP was synthesized as in [17] and [^{32}P]NAD as in [18]. Protein was determined as described [19] with modifications [20]. The $s_{20,w}$ values and Stokes' radii are given as means ± SD. Statistical analysis was performed using an unpaired *t*-test.

3. RESULTS

The influence of temperature on the sedimentation behavior of the cytosolic α-subunit is shown in fig.1. The $s_{20,w}$ value obtained at 30°C (fig.1B) amounted to 3.27 ± 0.18 S (n = 6). This value is consistent with the protein sedimenting as a monomeric species (see also [8]). The $s_{20,w}$ value obtained at 4°C (fig.1A) was 4.12 ± 0.12 S (n = 6), i.e. similar to that found for heterotrimeric G-proteins [4]. The difference between the values obtained at 4°C vs 30°C was highly significant ($P <$ 0.0001). The [^{32}P]ADP-ribosylated α-subunit exhibited the same mobility on SDS gels after treatment at 30°C as compared to treatment at 4°C (not shown). Thus, proteolytic cleavage is unlikely to be responsible for the different sedimentation behavior at the two temperatures. In addition, we found that the effect of a 90 min incubation at 30°C on the sedimentation behavior was completely reversed by incubation at 4°C for 23 h (two experiments, not shown).

To elucidate the nature of the different forms of the cytosolic G-protein α-subunit at the two temperatures, reconstitution experiments with $\beta\gamma$-complex purified from porcine brain were performed. We demonstrated recently that reconstitution of the cytosolic α-subunit with purified $\beta\gamma$-complex at 30°C resulted in a significant shift ($P < 0.001$) of the $s_{20,w}$ value from 3.27 ± 0.18 S (n = 6) to 4.04 ± 0.15 S (n = 5) [13]. This change probably represents the formation of a heterotrimeric G-protein. In fig.2 we show that at 4°C the addition of $\beta\gamma$-complex did not alter the $s_{20,w}$ value of the cytosolic α-subunit [4.12 ± 0.12 S (n = 6) and 4.18 ± 0.10 S (n = 4) in the absence and presence of $\beta\gamma$-complex, respectively]. However, $\beta\gamma$-complex greatly improved the susceptibility to pertussis toxin-catalyzed [^{32}P]ADP-ribosylation [13] (compare fig.2 with fig.1A). This suggests that the $\beta\gamma$-complex interacts with the cytosolic α-subunit at 4°C. The results further indicate that the cytosolic α-subunit at 4°C is not a heterotrimeric G-protein as might be deduced from its $s_{20,w}$ value.

Incubation of the cytosolic α-subunit with 10 μM GTPγS and 10 mM $MgCl_2$ (fig.3) resulted in a shift of $s_{20,w}$ values from 4.12 ± 0.12 S (n = 6) (fig.3A) to 2.93 ± 0.12 S (n = 3) (fig.1A) at 4°C and from 3.27 ± 0.18 S (n = 6) (fig.3B) to 2.38 ±

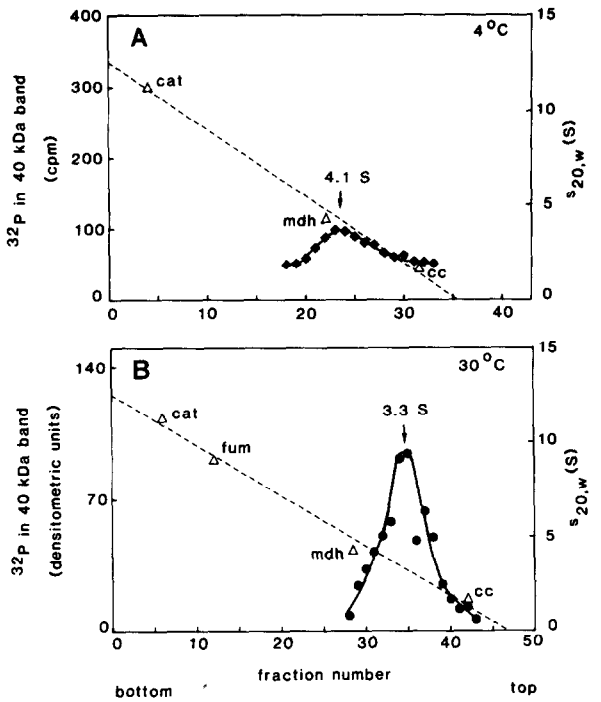

Fig.1. Sedimentation behavior of the cytosolic α-subunit at 4°C (A) and 30°C (B). The experiments were performed as described in section 2. The samples and the sucrose density gradients contained 50 μM GDP (which was added to stabilize the G protein) and 1 mM EDTA. The radioactivity incorporated into 40 kDa proteins is given in cpm (A, ♦) or arbitrary densitometric units (B, ●). In the experiments shown, $\beta\gamma$-complex was not added prior to ADP-ribosylation. Abbreviations of marker proteins (Δ) in this and the following figures are: cat, catalase; fum, fumarase; mdh, malic dehydrogenase; cc, cytochrome *c*.

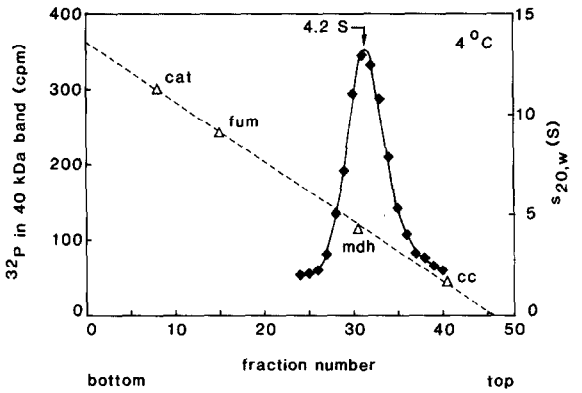

Fig.2. Reconstitution of the cytosolic α-subunit with purified $\beta\gamma$-complex at 4°C. Cytosol (120 μg protein) was incubated with $\beta\gamma$-complex (480 ng, purified from porcine brain) for 15 h at 4°C and subjected to centrifugation at the same temperature. The $\beta\gamma$-complex preparations used were free of pertussis toxin substrates as tested by pertussis toxin-catalyzed [^{32}P]ADP-ribosylation prior to and following centrifugation through sucrose density gradients. For explanation of symbols, see legend to fig.1.

0.10 S (n = 4) (fig.1B) at 30°C. The shift was highly significant at both temperatures ($P < 0.0001$ and $P < 0.001$ at 4°C and 30°C, respectively). The sedimentation coefficients obtained in the presence of GTPγS and $MgCl_2$ at 4°C and at 30°C were significantly different ($P < 0.001$). Furthermore, the values obtained in the presence of 10 μM GTPγS and 10 mM $MgCl_2$ at 4°C were significantly lower than those obtained at 30°C in the presence of 50 μM GDP ($P < 0.03$). The observed changes in sedimentation behavior suggest that the $\beta\gamma$-complex-free α-subunit undergoes a conformational change or is associated with an as yet unidentified protein, which is released upon activation with 10 μM GTPγS and 10 mM $MgCl_2$. The $s_{20,w}$ value obtained at 30°C (2.4 S) is similar to that reported for activated *and* dissociated G_i α-subunits [4].

To determine the amount of detergent bound to the cytosolic α-subunit, we have run sucrose density gradients in H_2O vs 94% (v/v) D_2O at 30°C (table 1). The sedimentation coefficient obtained in D_2O in the presence of 50 μM GDP (2.48 ± 0.21 S; n = 4) was significantly smaller than the corresponding one obtained in H_2O (3.27 ± 0.18 S; n = 6; $P < 0.001$), suggesting that a significant amount of detergent is bound to the cytosolic α-subunit. Estimations according to the method in [21] revealed that 0.58 mg Lubrol PX was bound per mg of protein (36.7% (w/w) detergent bound). In the presence of 10 μM GTPγS and 10 mM $MgCl_2$, the sedimentation coefficient obtained in D_2O-containing gradients (1.53 ± 0.17 S; n = 4) was also significantly lower that that obtained in H_2O-containing gradients (2.38 ± 0.10 S, n = 4; $P < 0.001$). We estimated that 0.89 mg detergent is bound per mg of protein (47.1% (w/w) detergent bound). Thus, the diminished sedimentation rate of the cytosolic α-subunit in the presence of 10 μM GTPγS and 10 mM $MgCl_2$ is probably not associated with a loss of bound detergent. The amount of Lubrol PX bound by the cytosolic α-subunit is significantly higher than the amount bound by the G_o α-subunit (0.1 mg Lubrol PX per

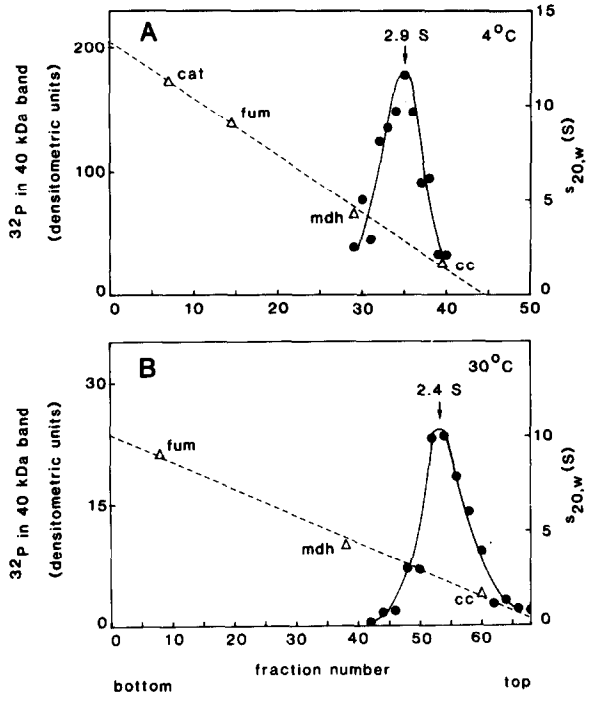

Fig.3. Activation of the cytosolic α-subunit with GTPγS and Mg^{2+} at 4°C (A) and 30°C (B). For activation of α-subunits, neutrophil cytosol was incubated with 10 μM GTPγS and 10 mM $MgCl_2$ (in the presence of 1 mM EDTA and the absence of GDP) for 2 h at 4°C or for 30 min at 30°C; centrifugation was performed at 4°C and 30°C, respectively. For explanation of symbols, see legend to fig.1.

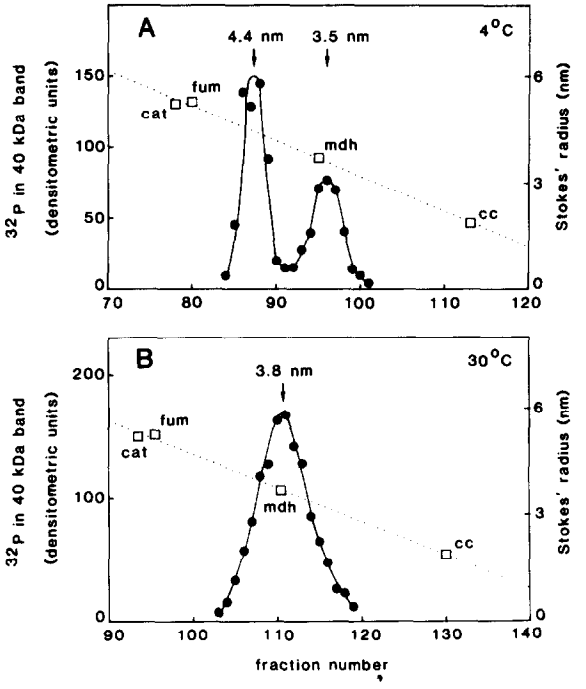

Fig.4. Gel filtration analysis of neutrophil cytosol on a TSK G3000SW column at 4°C (A) and 30°C (B). For experimental details see section 2. The radioactivity incorporated into 40 kDa proteins is given in arbitrary densitometric units. The position of marker proteins with known Stokes' radii is indicated (□).

Table 1

Detergent-binding properties of the cytosolic G-protein α-subunit

Molecular parameter	50 μM GDP, 1 mM EDTA	10 μM GTPγS, 10 mM $MgCl_2$, 1 mM EDTA
Sedimentation behavior		
Apparent $s_{20,w}$ in H_2O ± SD (n)	3.27 ± 0.18 S (6)	2.38 ± 0.10 S (4)
Apparent $s_{20,w}$ in D_2O ± SD (n)	2.48 ± 0.21 S (4)	1.53 ± 0.17 S (4)
Corrected $s_{20,w}$[a]	3.37 S	2.49 S
Partial specific volume $\bar{\nu}$[a]	0.840 cm^3/g	0.817 cm^3/g
Lubrol PX bound (mg/mg protein)[a,b]	0.58	0.89
Lubrol PX bound (in % of protein-detergent complex)[b]	36.7%	47.1%

[a] Calculated by the method of Sadler [21], using all possible paired combinations from H_2O and D_2O gradients

[b] Calculated using $\bar{\nu}_{protein}$ = 0.735 cm^3/g and $\bar{\nu}_{Lubrol}$ = 0.958 cm^3/g [24]

All determinations were performed at 30°C

mg of protein) [6], which has been calculated by the same method [21]. The relatively high amount of bound detergent indicates that the cytosolic α-subunit possesses hydrophobic regions. This may account for the tendency of the cytosolic α-subunit to aggregate under certain conditions in the absence of detergent: the $s_{20,w}$ value in the absence of detergent at 30°C was unexpectedly higher than the corresponding one in the presence of 0.1% (w/v) Lubrol PX (4.25 ± 0.06 S (n = 4), vs 3.27 ± 0.18 S (n = 6), $P < 0.0001$), whereas the sedimentation coefficient in the absence of detergent at 4°C was similar to that in the presence of detergent (4.30 ± 0.18 S (n = 4) vs 4.12 ± 0.12 S (n = 6)).

In contrast to the cytosolic G_{i2} α-subunit of human neutrophils, heterotrimeric G_i purified from human erythrocytes (which has been shown to contain both the G_{i3} and to a lesser extent the G_{i2} α-subunits) did not show a temperature-dependent alteration in its $s_{20,w}$ value under non-activating conditions (4.00 ± 0.24 S at 4°C (n = 4) vs 3.93 ± 0.15 S at 30°C (n = 3); not shown).

Gel filtration analysis of cytosolic proteins in the absence of detergent at 4°C and 30°C (fig.4A and B, respectively) revealed two peaks of [^{32}P]ADP-ribosylated 40-kDa proteins at 4°C as Stokes' radii were 4.38 ± 0.03 nm (n = 3) and 3.52 ± 0.06 nm (n = 3). At 30°C, a single peak was observed, corresponding to a Stokes' radius of 3.80 ± 0.17 nm (n = 3). These findings may indicate a monomer-dimer equilibrium at 4°C, while at 30°C the monomeric form prevails. The descending part of the curve shown in fig.1A, the sedimentation analysis at 4°C in the presence of 0.1% (w/v) Lubrol PX, is flattened towards the right and may represent a small portion of a monomeric α-subunit, which is detectable as a separate species by gel filtration analysis.

4. DISCUSSION

Human neutrophil cytosol contains a G-protein α-subunit devoid of $\beta\gamma$-complex, which by immunological criteria represents the G_{i2} α-subunit. The sedimentation behavior at 30°C is consistent with that of a monomer ($s_{20,w}$ value of 3.3 S). The $s_{20,w}$ value at 4°C (4.1 S) is compatible with (but not indicative of) that of an α_2-dimer. Evidence for α_2-dimer formation has been provided by sedimentation equilibrium analysis for the α-subunit of transducin [22] and by small angle neutron scattering analysis for the soluble polypeptide elongation factor Tu, EF-Tu, which – like G-protein α-subunits – binds and hydrolyzes GTP [23]. Alternatively, the α-subunit of neutrophil cytosol may be associated with an as yet unidentified protein at 4°C.

Several groups have determined $s_{20,w}$ values of isolated G-protein α-subunits. The α-subunit of G_o was found to sediment with an $s_{20,w}$ value of 2.8 S in the presence of detergent [6] and the α-subunit of transducin with an $s_{20,w}$ value of 3.4 S in the absence of detergent [7]. The $s_{20,w}$ values of $r\alpha_{s\text{-}S}$ and $r\alpha_{s\text{-}L}$, the recombinant α-subunits of the small and large form of G_s, were determined as 3.5 S in the absence of detergent [8].

A change in the sedimentation behavior upon activation was demonstrated previously for heterotrimeric G-proteins [3,4]. We report here that addition of 10 μM GTPγS and 10 mM $MgCl_2$ at 4°C and at 30°C causes a change in the sedimentation coefficient of an α-subunit devoid of $\beta\gamma$-complex. The change in sedimentation behavior may represent a conformational change associated with activation or, far less likely, dissociation of the α-subunit from an unidentified protein.

Codina et al. [4] reported that the $s_{20,w}$ value of heterotrimeric G_i changed upon addition of 10 μM GMP-P(NH)P and 10 mM $MgCl_2$ from 4.1 S to 3.3 S at 4°C. Following incubation and centrifugation in the presence of GMP-P(NH)P and $MgCl_2$ and at 30°C, α-subunits exhibited $s_{20,w}$ values of 2.2 S. Taking into account results obtained by gel filtration analysis, they suggested that both the '4 S' and the '3 S' forms represent $\alpha\beta\gamma$-heterotrimers, while the '2 S' form represents monomeric α-subunits. Our finding that the cytosolic α-subunit, which is devoid of $\beta\gamma$-complex, responds to treatment with GTPγS and $MgCl_2$ with a decrease in the sedimentation coefficient is in agreement with the hypothesis proposed by Codina et al. that subunit activation and dissociation can be identified individually.

A model depicting the different states of the cytosolic α-subunit described in this paper is shown in fig.5. Treatment of the cytosolic G-protein α-subunit with GTPγS and $MgCl_2$ at 4°C results in a conformational change of the α-subunit. However, this change may not be suffi-

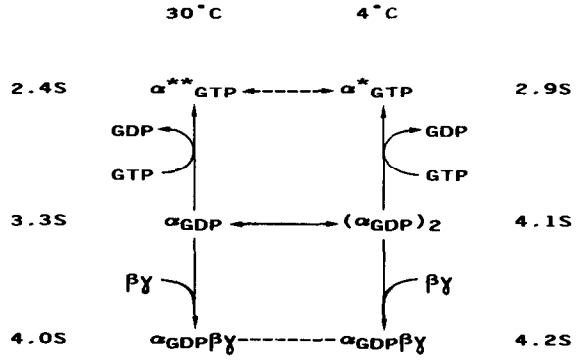

Fig.5. Conformational states of the cytosolic G-protein α-subunit of human neutrophils. Solid arrows, transitions shown experimentally, using GTPγS as a poorly hydrolyzable GTP analogue; dashed arrows, transitions not shown experimentally; * and **, activated forms of the α-subunit which may correspond to the '3 S' and '2 S' forms, respectively, obtained with heterotrimeric G-proteins [4]. The $s_{20,w}$ values of the various conformational states are depicted on the left and right margins. The $\alpha_{GDP}\beta\gamma$ forms at 4°C and 30°C do not differ significantly from each other by statistical analysis (4.18 ± 0.10 S (n = 4) vs 4.04 ± 0.15 S (n = 5) respectively).

cient to promote the dissociation of a heterotrimeric G-protein. Only upon incubation at 30°C in the presence of GTPγS and $MgCl_2$, a further conformational change, evident from a decrease in the $s_{20,w}$ value from 2.9 S to 2.4 S occurs. Thus, not only the 4 S to 3 S but also the 3 S to 2 S transitions described by Codina et al. [4] may reflect, at least in part, conformational changes inherent to the α-subunit.

Acknowledgements: We thank Susanne Brendel, Ingeborg Reinsch and Bettina Schein for expert technical assistance. This work was supported by the Deutsche Forschungsgemeinschaft, by the Fonds der Chemischen Industrie and by a Cusanuswerk predoctoral fellowship to U.R.

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